

SELECTION OF A HIGH ANTHOCYANIN-PRODUCING CELL LINE FROM CALLUS CULTURES OF HYBRID POPLAR (*POPULUS ALBA* L. × *P. GLANDULOSA* UYEKI)

Myung Suk Choi¹ & Young Goo Park²

¹) Division of Biotechnology, Forest Genetics Research Institute, P. O. Box 24, Kwonseon-gu, Suwon, 441–350, Korea

²) Department of Forestry, Kyungpook National University, Daegu, 702-701, Korea

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ABSTRACT

The reddish callus was visually selected from 15 callus lines based on color density. Anthocyanin synthesis ability was significantly different among 15 callus lines investigated. Line number 11 was considered as a reliable callus line for both high anthocyanin yield and rapid cell growth. Line number 11 frequently lost its ability for anthocyanin synthesis during repeated subculture and it did not provide reliable cell lines which produce high anthocyanin synthesis and cell growth. Callus derived from single cell colony was reselected by the cell aggregate selection method efficiently. The yield of anthocyanin from selected cell lines were increased almost two times (from 0.8 to 1.7) after the final selection (after 17 weeks). Anthocyanin content markedly increased from the 7th week but cell growth slightly decreased with selections. The content of anthocyanin from homogeneous callus lines varied from 0.8 to 1.7 per 0.5 g fresh weight of cultures.

Key words: *Populus alba* × *P. glandulosa*, anthocyanin, cell culture

INTRODUCTION

Plant secondary metabolites have been known to play a major role in the quality of food, flavour and dye, and insecticide (VERPOORTE *et al.* 1993). Plant cell cultures could be an alternative approach to the production of such secondary metabolites. Although in the past years all economically important plants have been brought into cell culture, in most cases the productivity was too low to allow an economically feasible process (MISAWA 1988). Selection of stable cell line and finding of optimum conditions for cell growth can solve these problems (VERPOORTE *et al.* 1993). However, many compounds which would be of value have not yet been isolated in sufficiently high yield. The most widely used selection methods is not very great, and the productivity of a selected cell line often is unsuitable because cells with different productivity exist in the original aggregate (CONSTABEL *et al.* 1981).

PARK *et al.* (1992) reported that production medium and growth medium for two stage cultures was devised from this hybrid poplar. Selection by using two stage cultures are available for strains which is not easy the maintenance for long time.

Anthocyanins are major secondary metabolites that have been studied in this species so far. Banning of synthetic red dyes in food products stimulated interests

in the development of pigments from natural sources. Although their primary role in the plant is related to coloration, roles in resistance to pathogens (WEISAETH, 1976) and pharmacological effects for man (BERETZ & CAZAHAVE 1988) also have been reported.

Hybrid poplar, *Populus alba* L. × *P. glandulosa* Uyeki, has been extensively planted in Korea since it was artificially bred by the Forest Genetics Research Institute of Korea in 1956. Those superior traits such as drought-resistance and fast growth than its parents made the hybrid a promising candidate for lumber production in short rotation in mountainous areas. Since cell culture can be established easily in this hybrid, it can serve as a model system for the production of useful secondary metabolites in tree species. The purpose of this study is to assess the problems of isolating stable, high yielding cell lines of *Populus alba* L. × *P. glandulosa* Uyeki.

MATERIALS AND METHODS

Visual selection

Callus derived from cambium tissues of *Populus alba* L. × *P. glandulosa* Uyeki were subcultured on MS (MURASHIGE & SKOOG 1962) medium containing 0.75% (w/v) agar, 3% (w/v) sucrose, 0.5 mg·l⁻¹ 2,4-

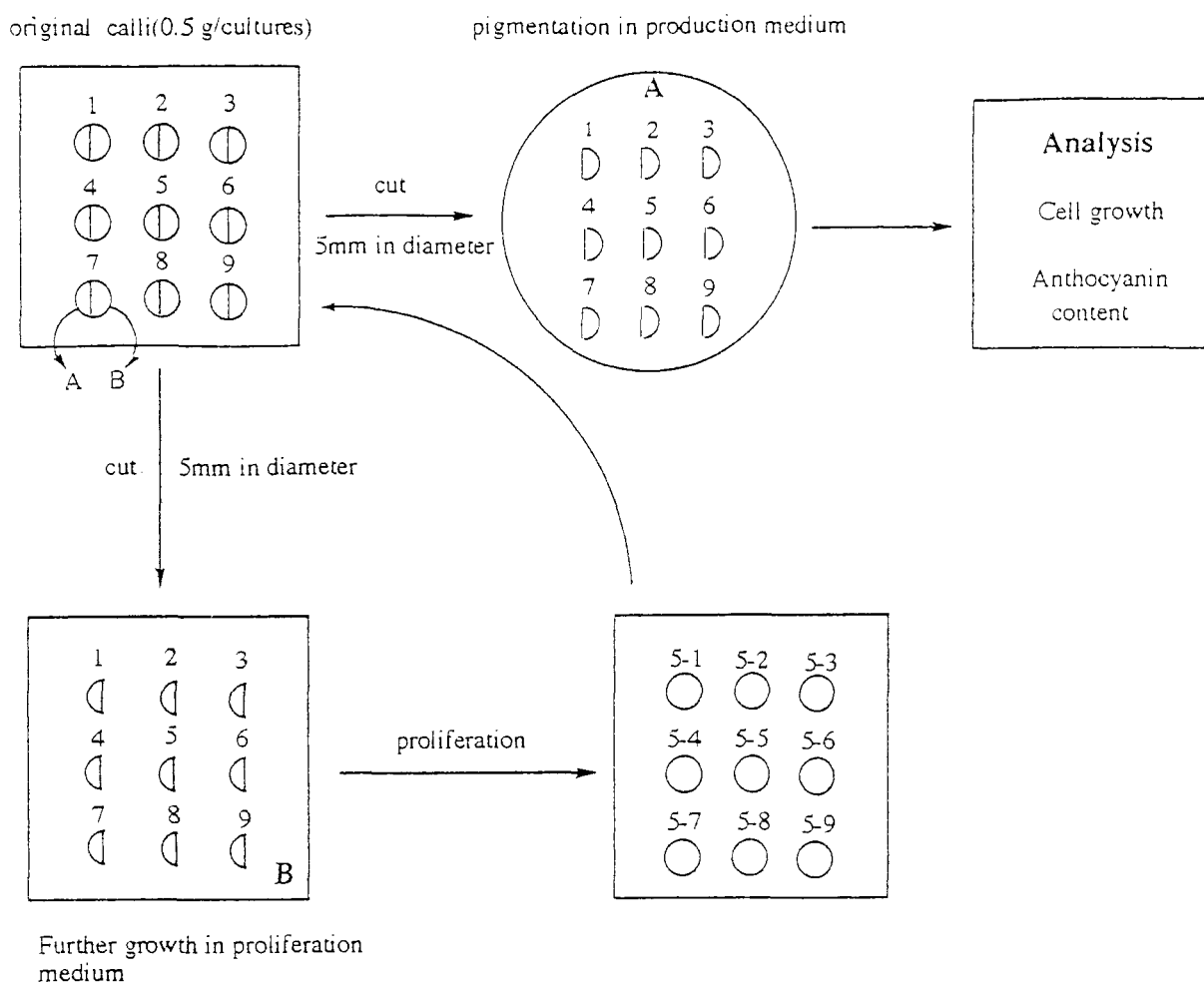


Figure 1. Schematic illustration of selection method of *Populus alba* × *P. glandulosa* Uyeki. Calli for anthocyanin synthesis (A) were cultured on MS agar medium supplemented with 1.0 mg·l⁻¹ IAA, 1.0 mg·l⁻¹ BA, and 5% sucrose under continuous illumination for 7 days. Calli for cell proliferation (B) were cultured on MS agar medium supplemented with 0.5 mg·l⁻¹ 2,4-D, 0.1 mg·l⁻¹ BA, and 3% sucrose under the dark condition.

dichlorophenoxyacetic acid (2,4-D), and 0.1 mg·l⁻¹ benzylaminopurine(BA). For visual selection, each callus pieces were maintained at 25 °C with a photosynthetically active photon flux rate of 60 Em⁻²S⁻¹ from cool white fluorescent tubes. Subcultures were made at 13 day intervals. Pigmented cells were counted under a microscope after separation of cells with 0.5% (w/v) cellulase "Onozuka R-10", 0.5% (w/v) Macerozyme (both from Kinki Yakult Ltd., Japan) and 0.02% (w/v) Pectolyase Y-23 (Seishin Ltd., Japan) as described previously (PARK & SON 1987). The anthocyanin content was determined by reading absorbance at 530 nm followed by the treatment reported by ARDITTE (1969).

Cell aggregate selection

Callus derived from single cell colony was reselected by the cell aggregate selection method as shown in Fig. 1. Callus was cut into two segments. Section A (ca. 5 mm in diameter) which was for anthocyanin synthesis was coded, arranged linearly, and then each segment was placed onto MS medium supplemented with 3% sucrose, 1.0 mg·l⁻¹ indole-3-acetic acid (IAA), and 1.0 mg·l⁻¹ BA (production medium) under fluorescent light of 60 Em⁻²S⁻¹. The other segment (section B), which was for cell proliferation was transferred onto a petri dish containing fresh MS medium supplemented with 0.5 mg·l⁻¹ 2,4-D and 0.1 mg·l⁻¹ BA (growth medium), and then cultured at 25 °C in the dark.

The section with the highest anthocyanin synthesis was selected by 17 successive clonal screening with 1 week interval as shown in Fig. 1

Anthocyanin identification

Extraction and identification of anthocyanins were followed by the modified ARDITTI (1969) methods.

RESULTS AND DISCUSSION

The reddish callus was selected from 15 callus lines based on color density. Both optimal cell growth and anthocyanin synthesis were observed on the MS medium containing 0.5 mg·l⁻¹ 2,4-D, 0.1 mg·l⁻¹ BA, and 3% sucrose. Anthocyanin occurred in the central vacuoles, frequently disappearing in the callus cells. Anthocyanin yield and cell growth of each cell line are shown in Table 1. Anthocyanin synthesis ability was significantly different among 15 callus lines investigated. Line number 11 was considered as a reliable callus line for both high anthocyanin yield and rapid cell growth. In this cell line, more than 80% of the cells contained anthocyanin. In contrast, only 33% of the cells in callus line 4 were observed to contain anthocyanin (data not shown).

Visually selected callus line number 11 frequently lost its ability for anthocyanin synthesis during repeated subculture. It was thus not reliable cell lines for high

anthocyanin synthesis and cell growth. After several subcultures, anthocyanin productivity of cells in the same clone became heterogeneous, maybe due to gene mutation and/or extrachromosomal variation observed in plastids of higher plants (MIZUKAMI *et al.*, 1987). This inconsistency can be improved by adopting cell aggregate selection.

The single cells were isolated by enzyme maceration from suspension cultured cells (Fig. 3.A). The first cell division of single cells was observed after 4 days of culture (Fig. 3.B), and calli were formed after 8 weeks

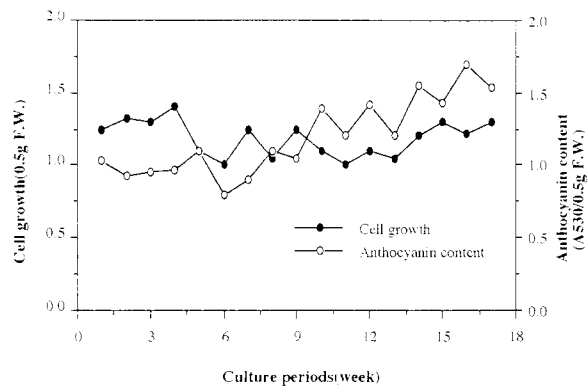


Figure 2. Changes in anthocyanin content during the serial subcultures *Populus alba* × *P. glandulosa* Uyeki. The callus for anthocyanin synthesis were cultured on MS agar medium supplemented with 0.1 mg·l⁻¹ IAA, 1.0 mg·l⁻¹ BA, and 5% sucrose under continuous illumination for 7 days. Each value represents the average of 5 replication, each in 3 independent experiments.

Table 1. Visual selection of a high anthocyanin producing calli lines of *Populus alba* L. × *P. glandulosa* Uyeki

Cell lines	Cell growth ¹	Color ³	Anthocyanin content
1	++ ²	Y	1.614 ± 0.512
2	+	W	0.824 ± 0.745
3	+	Y.B	1.056 ± 0.353
4	+	W	0.756 ± 0.242
5	++	Y	1.359 ± 0.356
6	+	W	0.883 ± 0.374
7	++	Y	1.419 ± 0.462
8	+	B	0.987 ± 0.328
9	+	Y.B	1.558 ± 0.487
10	++	Y.B	1.201 ± 0.385
11	+++	Y	2.277 ± 0.367
12	++	Y.B	1.405 ± 0.481
13	+	Y	1.309 ± 0.373
14	++	Y.B	1.322 ± 0.292
15	+++	Y.B	1.095 ± 0.290

¹⁾ Calli were cultured on MS basal medium supplemented with 0.5 mg·l⁻¹ 2,4-D, 0.1 mg·l⁻¹ BA, and 3% sucrose under continuous illumination.

²⁾ The number of + sign indicates the extent of cell growth (+: poor, ++: good, +++: very good).

³⁾ The capital character represents the colors of the callus (Y: Yellow, W: White, Y.B: Yellowish Brown, B: Brown)

(nonselected) of *Cytisus scoparius*. By the cell aggregate selection method, about two fold increment of yields was obtained. However, further experiments on the serial selection are still needed to warrant this system. Results described in this paper may help to overcome obstacles involved in plant secondary metabolite production such as low and instable productivity. And this study can serve as a model system in the study of useful secondary metabolites from tree species which is recalcitrant to in vitro manipulation. This system for selecting high anthocyanin yield cell lines appears to be readily adaptable for other tree species aimed at the commercially attractive biosynthesis of secondary metabolites.

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